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13. ABSTRACT ***(Maximum 200 Words)***

We have developed data demonstrating that the pineal gland, via its hormone melatonin, inhibits the proliferation of both human and animal models of breast cancer. As humans age there is the onset of disrupted sleep leading to a significant suppression in the nocturnal levels of melatonin after age 60. Based on these data we have hypothesized that the decline in pineal melatonin production, with the onset of old age, is a key factor in the age related increase in breast cancer. Using the Buffalo rat as a model, we have begun to characterize the melatonin rhythm in young, middle aged and old female rats. Our studies demonstrate that the nocturnal rise in both serum and pineal melatonin is significantly blunted in old rats compared to middle aged and young rats, and is blunted in middle aged rats compared to young rats. As well, uterine mt1 melatonin receptor levels are greatly diminished in old female rats (by 80%) compared to young female rats. Finally, in our preliminary studies, tissue-isolated NMU-induced mammary tumors grew faster in young rats as compared to middle aged rats. However, tumors in middle aged rats are less responsive to the growth-suppressive actions of melatonin.

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INTRODUCTION:

An increasing percentage of elderly women, particularly in industrialized countries, are developing breast cancer. Furthermore, it is expected that breast cancer incidence will continue to increase with increased age. A number of hypotheses have been proposed to explain this dramatic increase in breast cancer incidence in the later stages of life, including total dose of carcinogen exposure. However, an alternative hypothesis is that aging results in changes in the internal milieu of the organism, such as metabolic, endocrine and immunologic shifts, providing increasingly favorable conditions for tumor induction, promotion and progression. The pineal gland, via its hormone melatonin, has been shown to play a central role in the regulation of circadian rhythms as well as sleep\wake cycles. Furthermore, the synthesis of melatonin, which is increased in response to darkness, can be blunted and even blocked by exposure to light at night. As individuals age, there is the onset of disrupted sleep and nighttime exposure to light, leads to a significant suppression in the nocturnal levels of melatonin after age 60. Over the last two decades, considerable evidence has accumulated demonstrating that the pineal gland, via its hormone melatonin, possesses significant oncostatic activity, particularly for breast cancer. Our studies have demonstrated that melatonin is able to significantly suppress the transcription of the ER gene, and that it modulates the expression of estrogen-regulated growth-stimulatory factors, oncogenes, and proteins (TGFα, *c-fos*, pS2 and PgR), as well as increasing the expression of the potent growth-inhibitor TGFβ, through the activation of a membrane associated G-protein-coupled receptor, the Mel1a (mt1) receptor. Therefore, given that melatonin levels diminish significantly by the 5th and 6th decades of life as the incidence of breast cancer increases, we hypothesize that *the age related decline in pineal melatonin production leads to an enhancement of breast cancer development and growth in older women*. Given that there are no well designed or tested models of aging and breast cancer the purpose of these studies is to define the age-related changes in melatonin and melatonin receptors, and thus, sensitivity to melatonin in response to age in the female Buffalo rat for the purpose of using the Buffalo rat transplantable mammary tumor model to test the above hypothesis. The scope of the research for the second year was to characterize age related changes in melatonin production and responsivity to melatonin in female Buffalo rats, and to define the growth of tumors in young vs. middle age vs. old female rats.

BODY:

Our studies have demonstrated that Buffalo rats are quite sensitive to the oncostatic effects of melatonin and to changes in photoperiod. However, the endogenous circadian rhythm of melatonin has not been fully characterized in this rat model. Given that responsiveness to exogenous melatonin is associated with endogenous melatonin synthesis and that the endogenous melatonin rhythm apparently changes with the onset of old age, it is essential that we fully characterize the differences in the circadian melatonin profiles in young, middle aged and old rats. To accomplish these goals we proposed the following Specific Aims for years 1 and 2.

1. **To characterize age related changes in melatonin production and responsivity to melatonin in Buffalo rats by:**
   a. Examining melatonin rhythms in young, middle aged, and old Buffalo rats.
   b. To characterizing the expression of the melatonin (mt1) receptor in the hypothalamus and uterus of young, middle aged, and old Buffalo female rats.
   c. Measuring responsivity to melatonin in young, middle aged, and old rats.
2. **To characterize melatonin's effects on the growth of transplantable N-nitroso-N-methylurea (NMU)-induced mammary tumors in young, middle aged and old female Buffalo rats by:**
   a. Comparing mammary tumor growth in young, middle aged and old rats.

To accomplish the studies proposed in this specific aim we proposed following Statement of Work: *Since we had to age our own rats, we have included data from the first year that we have now filled out with data from old rats (20 months of age).*

First Six Months our tasks, as outlined in the grant were:

- To purchase young, middle aged and old Buffalo rats, and let them adjust to long day photoperiod (12L:12D), then collect serum and measure melatonin serum levels. These studies will define the differences in melatonin levels in young, middle aged and old rats that will serve as the baseline for future studies.

First Year:

- Determine the differences in serum melatonin levels in young, middle aged and old Buffalo rats.
- Characterize differences in melatonin receptor (mt1) expression in melatonin-responsive tissues (uteri) in young, middle aged and old Buffalo rats.

Second Year:

- Define which animals young, middle aged or old, are more responsive to exogenous melatonin by measuring ER, TGFβ and RARα and β expression in hypothalami and uteri of melatonin-treated rats.
- Determine the growth characteristics of tissue-isolated transplanted NMU mammary tumors in young, middle aged and old Buffalo rats, and define the molecular and cellular characteristics of these tumors in the different age groups.

For these studies we purchased female Buffalo rats, BUF (BUF/Ner) (National Cancer Institute) from Charles River Laboratories (Kingston, NY) at 4 weeks of age and maintained in environmentally controlled rooms in facilities (Tulane Vivarium). After 4 weeks in long day photoperiod (12L: 12D) two groups of Buffalo rats (10 rats in each group), at 2 month, 15 months of age, *and now 20 months of age*, corresponding to young, middle aged (adult), *and old* rats, respectively, were exsanguenated and truncal blood collected during the light phase (at 0900 and 1600 h), the dark phase (1800, 2000, 2300, 2400, 0100, 0200 and 0400 h) and then again at 0900 h. During the dark phase, blood samples were collected under a dim red light (Kodak Safelight) to avoid light-induced suppression of melatonin production (1). Melatonin levels were measured in the serum over a 24 h period using an ultrasensitive RIA for melatonin (2). We have used this assay

previously for the measurement of melatonin levels in Buffalo rats (3). Data from these studies were analyzed by ANOVA simultaneously accounting for sources of variation principally conceived as treatments and time, with repeated measures where indicated.

One problem developed with this project that we had not anticipated. The supplier of the Buffalo rats, Harlan Sprague-Dawley, no longer maintains aged (20 – 30 month old) rats. Therefore, we have had to purchase these animals and have begun to age them. *As some of these rats are just now reaching old age (20 months or greater), we are beginning to examine both their serum and pineal melatonin levels. Thus, we now have examined young (2 mo), adult (15 mo), and old (20 mo) female rats with regards to serum and pineal melatonin levels and mt1 melatonin receptor levels in the uterus.* We do anticipate that we will be able to complete the entire project (young, adult and aged rats) close to the proposed deadline.

<u>Serum levels of melatonin:</u> Our data as shown in Figures 1 and 2 demonstrate that in female Buffalo rats nocturnal serum melatonin levels diminish significantly from young rats (8 weeks of age) to adult rats (15 months of age) and *even to a greater extent in old (20 months of age) rats.* Figure 1 shows the diurnal rhythm of serum melatonin in young, middle age and old rats. As shown in this figure, a significant difference in the timing of the onset or offset of melatonin serum levels is evident between young and adult rats. With lights off at 1800 h (6:00 p.m.) and on at 0600 h (6:00 a.m.) melatonin levels in young rats began to rise between 1800 h and diminish, back to day time values, by 0500 h. In adult rats the onset of melatonin levels during the dark phase of the light:dark cycle was delayed to approximately 2000 h and returned back to daytime values by 0400 h. Thus, adult rats showed at 2-3 h reduction in the plateau of melatonin production. This decrease in the length of the plateau of melatonin was also accompanied by a significant ($p < 0.05$) decrease (29%) in the peak value of serum melatonin as shown in Figure 2. In young rats the mean peak serum levels of melatonin was 123 pg/ml of serum, while in adult rats mean peak serum levels of melatonin was 88 pg/ml. *In old rats (20 mo.) the onset of melatonin levels during the dark phase of the light: dark cycle was delayed to approximately 2250 h resulting in a 3-5 h reduction in the length of the melatonin plateau compared to young animals. As well the old female rats showed a highly significant diminution of peak serum melatonin levels from 123 pg/ml of serum in the young to 30 pg/ml of serum.*

Figure 1. Changes in female Buffalo rat melatonin rhythm with increasing age. Serum melatonin levels of 10 young (2 months of age), 10 adult (15 months of age) *and 10 old (20 months of age)* female Buffalo rats at time points of 0900 and 1600 h (light phase) and 1800, 2000, 2300, 2400, 0100, 0200 and 0400 h (dark phase). Animals were maintained in a light:dark cycle of 12:12 before being killed. The curve obtained for both ages is roughly sinusoidal, with low levels during the daytime ☐ and elevated levels at nighttime ■ .

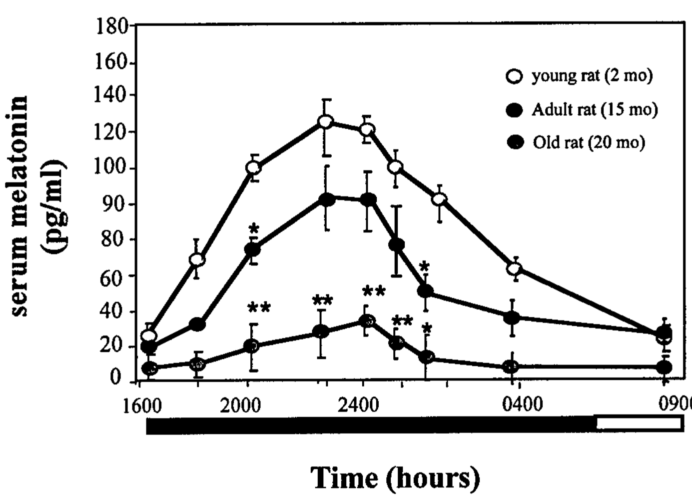
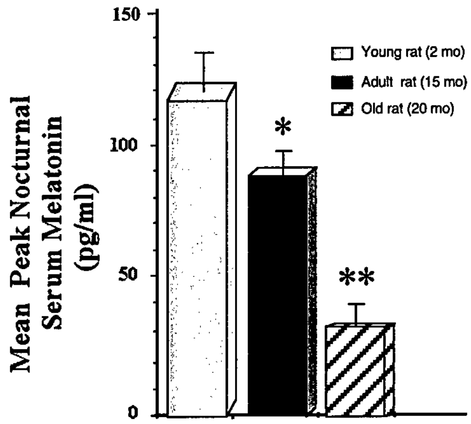
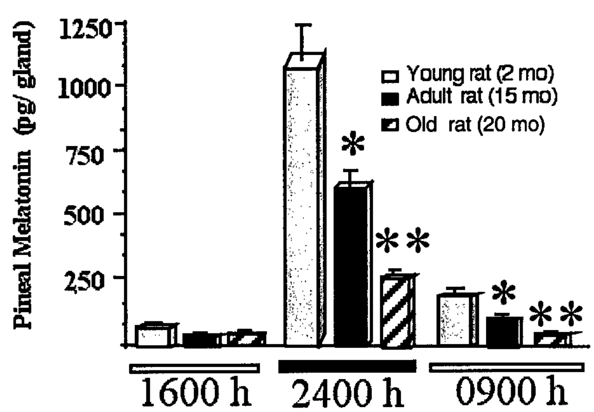


Figure 2. Peak daytime and nighttime serum levels of melatonin in adult and young female Buffalo rats. Serum melatonin levels of 10 young (2 months of age),10 adult (15 months of age) and old *(20 months of age)* female Buffalo rats at 2400 h (dark phase). Animals were maintained in a light:dark cycle of 12:12 before being killed under a red-light. * $p<0.05$ vs. young rats.



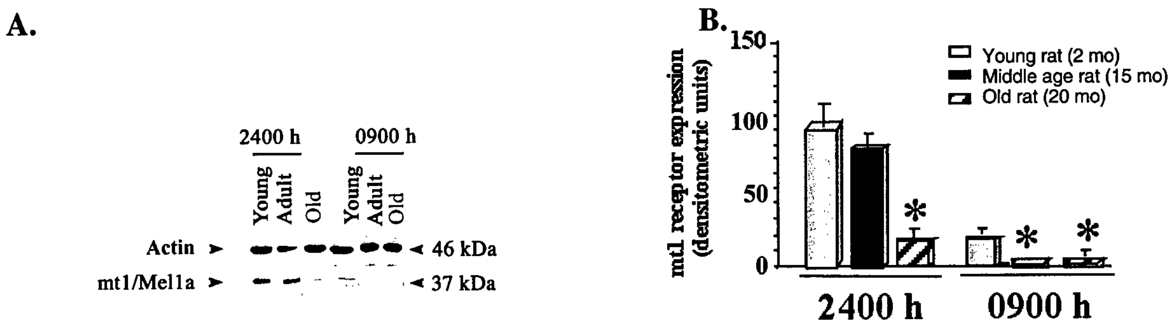
Pineal melatonin levels: In addition to examining changes in serum melatonin levels in young and adult Buffalo rats, we also examined pineal levels of melatonin in these same animals. Again, animals were kept in long day photoperiod on a 12 light: 12 dark cycle with lights off at 1800 h (6:00 p.m.) and on at 0600 h (6:00 a.m.). After 4 weeks in long day photoperiod (12L: 12D) three groups of Buffalo rats (10 rats in each group), at 2 month, 15 months of age and *20 months of age*, corresponding to young, adult and *old rats*, respectively, were exsanguenated and during the light phase (at 0900 h) and the dark phase (2400 h). During the dark phase (2400 h), pineal samples were collected under a dim red light (Kodak Safelight) to avoid light-induced suppression of melatonin, frozen on solid CO_2. Pineal glands were then stored frozen at –20° C until melatonin assays were performed 3-10 days later. Melatonin levels were measured in the serum over a 24 h period using an ultrasensitive RIA for melatonin as described above. Figure 3 shows daytime and nighttime pineal melatonin levels in young (2 months), middle aged (15 months of age) and old (20 months of age) female rats. Middle aged adult rats in this study showed a significant ($p<0.01$) diminution of nighttime pineal melatonin levels compared to young rats, while old age rats showed a highly significant diminution of pineal melatonin compared to both young and middle age rats. At this time, the melatonin content of the pineal glands of the young rats exceeded daytime levels by 13-fold, where as in the middle aged, adult, rats only a 7-fold increase in nocturnal levels of pineal melatonin were observed. *In the old rats only a 3-fold increase in nocturnal levels of pineal melatonin were observed.*

Figure 3. Pineal melatonin content in young and adult female Buffalo rats. Pineal melatonin levels of 7 young (2 months of age), 10 adult (15 months of age) *and old (20 mo)* female Buffalo rats collected at 2400 h (dark phase). Animals were maintained in a light:dark cycle of 12:12 and were exsanguenated under a dark -light. * $p<0.05$ vs. young rats.



<u>Uterine expression of the melatonin mt1 receptor:</u> It has been well demonstrated that melatonin can modulate uterine function. For example, female hamsters placed in long day photoperiods will have fully developed uteri and administration of melatonin, late in the afternoon, will induce the involution of the uterus. As shown in Figure 4, female Buffalo rats express quantifiable levels of the melatonin mt1 receptor. This figure also shows that uterine levels of the mt1 receptor are diminished by 41% in middle age adult rats *and by 80% in old rats (20 months of age)* compared to young rats.

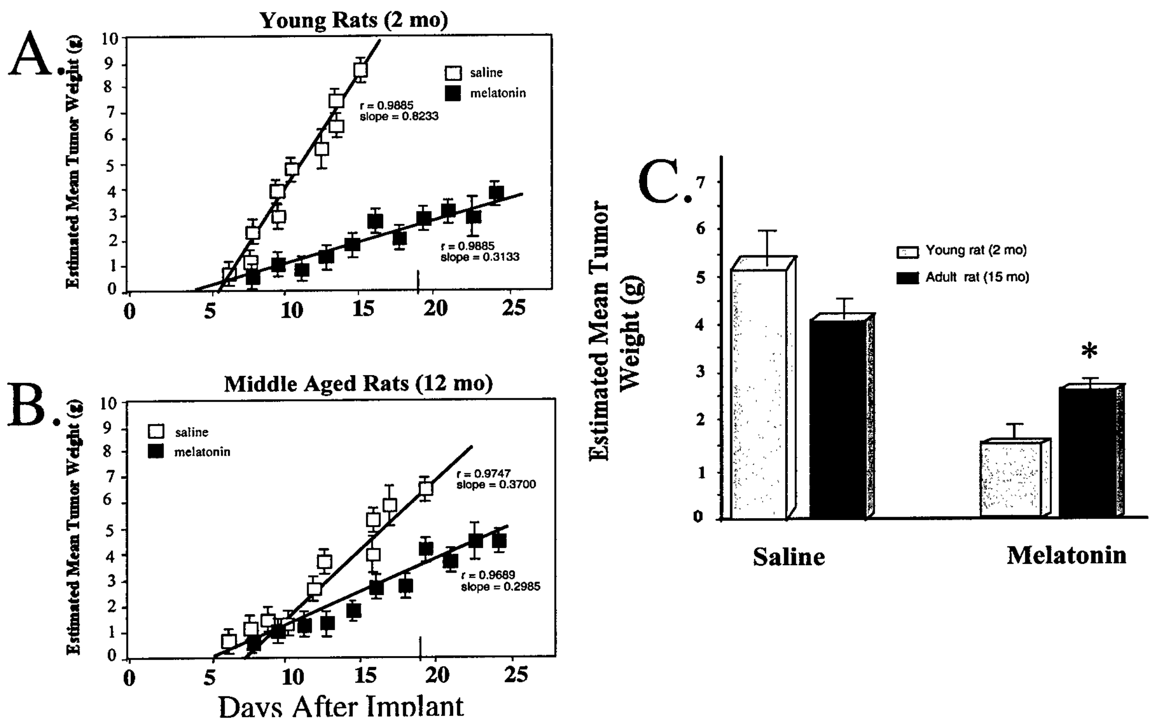
Figure 4. Uterine melatonin mt1 receptor expression in young and adult female Buffalo rats. (A) Melatonin mt1 receptor protein levels were measured by Western blot analysis, using the 563 anti-mt1 antibody, provided by Dr. Rolf Jockers (Paris, France), from total cellular protein isolated from the uteri of 10 young (2 months of age), 10 adult (15 months of age) and 4 old (20 months of age) female Buffalo rats at 2400 h (dark phase) and 0900 h (light phase). Animals were maintained in a light:dark cycle of 12:12 and a red-light was used when animals were exsanguenated during the dark phase. (B) Densitometric analysis of mt1 Westerns from 4-10 different animals in each group. * p<0.05 vs. young rats.



<u>Tumor Growth as a Function of Age and Melatonin Treatment:</u> It has been well documented that melatonin can significantly suppress the growth of N-nitroso-N-methylurea (NMU)-induced mammary tumors in rats (1). Using a tissue-isolated transplantable model tumor that we have previously employed (3), we examined the growth of NMU-mammary tumor transplants in response to age or melatonin as a function of host age. Briefly, a 3-mm cube of tumor was sutured to the tip of the vascular stalk formed from the superficial epigastric artery and vein. The tumor implant and vascular stalk were enclosed within a parafilm envelope and placed in the inguinal fossa, and the skin incision closed. Vascularization of the implant is limited to new vessel connections with the epigastric artery and vein: subsequent tumor growth is s.c. When tumors reached sufficient size for measurement, rats were subjected to light CO_2 narcosis and tumor dimensions were measured through the skin with vernier calipers and were converted to tumor weights as described previously (3). Growth rates (g/day) were generated by linear regression from the estimated tumor weights for the treated and control animals. The final tumor weight was determined by weighing at the end of the experiment. As noted in this figure, the growth of tissue-isolated tumor implants was greatest in young rats as was the growth-suppressive response of tumors to exogenous melatonin-administration. The average tumor after 25 days of growth, as shown in Figure 5C, was estimated at 5.1 g in the young rats vs. 3.8 g in the middle aged rat. As well tumor growth was inhibited by 71% in young rats as compared

to only 37% in middle aged rats in response to exogenous melatonin. Thus, a significant suppression in responsivity to melatonin was noted in the middle-aged rats as compared to young rats.

Figure 5 Effects of age and melatonin on the growth of tissue-isolated NMU-induced mammary tumors in Buffalo rats. (A) Tumor growth in young rats (2 mo.) and (B) tumor growth in middle aged rats (15 mo.) in response to diluent (0.1 ml of ethanolic saline) or melatonin (200 μg/0.1 ml) injections administered every day in the late afternoon (4:00-6:00 p.m.) to rats provided with a semipurified diet containing 5% corn oil *ad libitum.* Regression analysis and tests for parallelism indicate that melatonin treatment significantly (* P<0.05) decreased the tumor growth rate in both the young and middle aged animals. (C) The average estimated tumor size after 25 days of growth between controls and melatonin treated rats in both young and middle aged rats.



KEY RESEARCH ACCOMPLISHMENTS:

- The onset and offset of the melatonin production was significantly delayed and retracted, respectively, in old rats compared to young and adult rats, so that the phase of melatonin production was significantly delayed in old rats compared to middle aged and young rats, and in middle aged rats compared to young rats.

- The peak nocturnal serum melatonin level was significantly blunted in old buffalo, as compared to young and adult rats, to the point that it was in some old rats it was not significantly greater than the day values seen in young rats.

- The nocturnal production of pineal melatonin was significantly blunted (by 2.6- and 4.5-fold) in the pineal glands of old (20 months) Buffalo rats compared to adult (15 month) and young (2 months) rats, respectively.

- The uterine levels of mt1 receptor were significantly diminished by 79% in uteri from old rats as compared to young and even middle aged rats.

- The growth rate of transplantable mammary tumors in middle age rats was moderately increased compared to young rats, while growth-suppression by melatonin was significantly reduced in middle age rats compared to young rats.

REPORTABLE OUTCOMES:

- Army/DoD report
- Abstract at the 2002 Era of Hope meeting in Orlando, FL

CONCLUSIONS:

The major question addressed in this project is whether melatonin levels and sensitivity are diminished with advancing age and if these changes make aged rats more susceptible to mammary tumor development. In our second phase of this project we have demonstrated that there is a significant 4-fold decline in peak serum melatonin levels in old female Buffalo rats compared to young rats. In addition, the period of melatonin release is significantly shortened in old as compared to middle age and young rats. These data correlate with the decline in pineal melatonin production in the old rats as compared to middle age and young rats. As well, we have demonstrated that in old rats, that the levels of the mt1 receptor is dramatically diminished (by almost 80%) in uterine tissues during the light phase of the light/dark cycle compared to both middle age and young rats. It is important to note that mt1 receptor levels are as well reduced in middle aged rats compared to young rats. Although the data for the middle age and young rats was generated last year, we now have added the critical data from the old rats (20 months of age). Thus, if melatonin does possess antitumor activity with regards to breast cancer, we would expect adult rats to be more susceptible to the formation of mammary tumors, based on their reduced levels of endogenous melatonin. Our early data with the tissue-isolated transplantable mammary tumors in young vs. middle age rats indicates that reduced melatonin and mt1 receptor levels in middle age rats allow tumors does not necessarily affect the overall growth rate of tumor, but does appear to make them less sensitive to the growth-suppressive actions of exogenous melatonin. Studies with tissue-isolated transplantable mammary tumors in old rats, will be completed in year 3 as more rats come of age.

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APPENDICIES:

Abstract for Era of Hope 2002

AGE RELATED DECLINE IN SERUM AND PINEAL MELATONIN LEVELS AND UTERINE MT1 MELATONIN RECEPTOR LEVELS IN YOUNG AND MIDDLE-AGED FEMALE BUFFALO RATS.

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Over the last two decades, considerable evidence has accumulated demonstrating that the pineal gland, via its hormone melatonin, possesses significant oncostatic activity, particularly for breast cancer. Our studies have demonstrated that melatonin is able to significantly suppress the transcription of the ER gene, and that it modulates the expression of estrogen-regulated growth-stimulatory factors (TGFα, TGFβ, *c-fos*, pS2 and PgR) through the activation of a membrane associated G-protein-coupled receptor, the MT1 melatonin receptor. As individuals age, there is the onset of disrupted sleep and nighttime exposure to light, leading to a significant suppression in the nocturnal levels of melatonin after age 60. Therefore, given that melatonin levels diminish significantly by the 5th and 6th decades of life as the incidence of breast cancer increases, we hypothesize that *the age related decline in pineal melatonin production leads to an enhancement of breast cancer development and growth in older women.* Given that there are no well designed or tested models of aging and breast cancer the purpose of these studies is to define the age-related changes in melatonin and melatonin receptors, and thus, the response of transplanted carcinogen-induced mammary tumors in the young, middle-aged and old female Buffalo rats.

To begin to define the melatonin profile in young, middle-aged and old female Buffalo rats, serum and pineal melatonin levels were measured in young (8 months of age) and middle-aged (15 months of age) rats. Serum melatonin was measured at 0900 and 1600 h (light phase) and 1800, 2200, 2300, 2400, 0100, 0200 and 0400 h (dark phase) of 10 young and 10 middle-aged female rats. Our data demonstrate that in female Buffalo rats nocturnal serum melatonin levels diminish significantly from young to adult rats, with a significant difference in the timing of the onset of melatonin serum levels evident between young and adult rats. In adult rats the onset of the evening melatonin rise was delayed by approximately 2-3 h. This delay in the onset of the melatonin plateau was also accompanied by a significant ($p < 0.05$) decrease (29%) in the peak value of serum melatonin (mean peak melatonin serum level of 123 pg/ml and 88 pg/ml of serum in young and adult rats, respectively) in middle-aged rats. The level of pineal melatonin was also examined in these same animals. Middle aged adult rats in this study showed a significant ($p < 0.01$) diminution of nighttime pineal melatonin levels compared to young rats. The nighttime melatonin content of the pineal glands of the young rats exceeded daytime levels by 13-fold, where as in the middle aged rats only a 7-fold increase in nocturnal level of pineal melatonin was observed.

It has been well documented that melatonin can modulate uterine function in hamsters. Our studies demonstrate that the uteri of female Buffalo rats express quantifiable levels of the MT1 melatonin receptor and that the levels of this receptor are diminished by 41% in adult rats compared to young rats. Currently, studies are underway comparing the tumor take and growth of N-nitro-N-methylurea (NMU)-induced tumors transplanted into young and middle-aged Buffalo female rats